Impact of Haptoglobin Gene Polymorphism on Phenotypic Variability in β-thalassemia Patients: Relation to Iron Overload and Oxidative Stress

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Background and study aim: Thalassemia syndromes are genetic disorders in globin chain production. There is excessive synthesis of reactive oxygen species in thalassemia which leads to oxidative stress. Excessive production of malondialdehyde (MDA) provides evidence that thalassemic erythrocytes contain a significant amount of membrane-bound iron. Inflammatory cytokines trigger the production of haptoglobin (Hp) in the liver. It is characterized by molecular heterogeneity brought on by genetic polymorphism. The work aimed to study the impact of haptoglobin gene polymorphism on phenotypic variability in thalassemia patients and its relation to iron overload and oxidative stress.

Patients and Methods: This study was carried out on 50 β-thalassemia major patients in addition to 25 healthy age and sex-matched individuals as control. All patients and control were subjected to: history taking, physical examination, Laboratory investigations, Iron profile, Serum ferritin, Serum haptoglobin (Hp), Serum malondialdehyde (MDA), and Haptoglobin gene polymorphism by (PCR).

Results: The study revealed a significant decrease in serum haptoglobin and a significant increase in MDA and ferritin levels in thalassemic patients compared to the control group. Higher incidence of Hp2-2 in the thalassemic group while the control group showed more prevalence of Hp2-1. Serum level of MDA was significantly higher and haptoglobin levels were significantly lower in patients with Hp2-2 genotype. Serum ferritin was higher in the same genotype but did not reach a significant value.

Conclusion: Haptoglobin polymorphism and phenotypic variability have a major influence on oxidative stress in thalassemia patients.

INTRODUCTION

Thalassemia syndromes are genetic disorders in globin chain production characterized by varying degrees of ineffective hematopoiesis and increased hemolysis. They range from asymptomatic forms to severe or even lethal entities, depending on the clinical severity **[1]**. The clinical phenotypes of β-thalassemia major (βTM) are remarkably distinct (homozygous or compound heterozygous states) having significant anemia from early life. The standard protocol for their treatment is lifelong blood transfusion and chelation therapy. Chronic blood transfusions, despite being lifesaving, lead to organ and tissue damage due to iron overload **[2]**. Due to its capacity to chelate transition metals and block their redox activity, the iron chelator deferoxamine (DFO) has been utilized for many years to prevent iron overload in thalassemia patients **[3]**.

There is excessive synthesis of reactive oxygen species in thalassemia. All of which leads to oxidative stress. Its primary cause is iron overload, which is brought on by increased gastrointestinal iron absorption, repeated blood transfusions, and ultimately iron release from heme. Iron is incriminated to the excessive production of free radicals that might

Damage erythrocytes through oxidative stress. The hastened apoptosis that could result from this oxidative stress may reduce the lifespan of erythrocytes **[4]**. Excessive production of malondialdehyde (MDA), a lipid peroxidation byproduct, provides evidence that thalassemic erythrocytes contain a significant amount of membrane-bound iron. Increased serum MDA and decreased antioxidant defense system are assigned to the peroxidative damage of lipids, which contributes to the pathogenesis of thalassemia **[5]**.

The acute phase protein Haptoglobin (Hp) lowers the oxidative and peroxidative potential of free hemoglobin and removes it in intravascular or extravascular hemolysis. It acts as an inhibitor of angiogenesis and prostaglandin synthesis and is produced in the liver in response to glucocorticoids and inflammatory cytokines **[6]**. Haptoglobin is recognized by molecular heterogeneity brought on by genetic polymorphism. There are three common phenotypes of Hp: Hp1-1, Hp2-2, and the heterozygous Hp2-1 which are controlled by two autosomal co-dominant alleles identified as Hp1 and Hp2. They differ in their functional efficiencies, and biophysical, and biochemical properties, which contribute to their varied antioxidant and immunomodulatory capacities. The peripheral blood B-cell and T-lymphocyte counts are higher with the Hp2 allele, but the anti-inflammatory effect is less prominent than with Hp1allele **[7]**. Haptoglobin polymorphism is accompanied by the clinical evolution and prevalence of many autoimmune disorders, atherosclerosis, and inflammatory diseases, including infection. The antioxidant function of haptoglobin and the phenotypic dependence were validated for inhibiting the potential oxidative damage caused by free hemoglobin and iron release during its breakdown **[8]**.

The present study aimed to study the effect of haptoglobin gene polymorphism on phenotypic variability in thalassemia patients and its relation to iron overload and oxidative stress.

PATIENTS AND METHODS

Fifty patients previously diagnosed with β thalassemia major were included in our study. They were on regular RBC transfusion and iron chelation therapy using deferoxamine (DFO). Blood samples were collected before blood transfusion. In addition, twenty-five healthy individuals were included as controls. After obtaining approval from the Ethics Committee, patients or their parents provided informed consent to participate in the study,

All patients as well as controls were subjected to: history taking, physical examination, and Laboratory investigations including Complete Blood Count (CBC), Reticulocyte count **[9]**, Hb electrophoresis **[10]**, Iron profile (serum iron, total iron binding capacity, transferrin saturation), Serum ferritin **[11]**, C- reactive protein (CRP) **[12]** and Coombs'test **[13]**. In addition to Serum haptoglobin (Hp) using an immune-nephelometry automated chemistry analyzer **[14]**, Serum malondialdehyde (MDA) by spectrophotometric thiobarbituric acid (TBA) test method **[15]** and Haptoglobin gene polymorphism by polymerase chain reaction (PCR) **[14]**.

Statistical analysis

IBM SPSS software package version 23.0 was used to analyze data (Armonk, NY: IBM Corp). The distributions of quantitative variables were tested for normality using Kolmogorov-Smirnov and Shapiro-Wilk tests. We evaluated normally distributed quantitative data by independent t-test and ANOVA while not normally distributed data were evaluated by Mann-Whitney and Kruskal-Wallis tests. Qualitative data were tested by the Chi-square and Fisher's exact tests. The level of significance was set as $p \leq 0.05$.

RESULTS

The study comprised fifty patients with established βTM. They were 22 (44%) males and 28 (56%) females, their ages ranged from (12.0-20.0 years) with a mean age of 16.38±2.65 years. Twenty-five healthy individuals were included as a control group, 15 (60%) females and 10 (40%) males, ranging in age from (13 to 20 years) with a mean of 16.40 ± 2.60 years.

On clinical examination, forty-two (84%) patients had hepatomegaly, twenty-two (44%) patients had undergone splenectomy, and eighteen patients (36%) had splenomegaly. On the other hand, sixteen (32%) patients had hepatitis C infection.

As regards CBC results, table 1 shows the hematological data of βTM patients & the control subjects. There was a significant decrease

in the mean Hb, PCV, MCV, MCH, and RBCs but the mean platelets and reticulocyte counts were significantly higher in thalassemia patients compared to controls (P<0.001).

Iron profile

The mean serum iron and transferrin saturation in addition to serum ferritin was significantly higher in thalassemia patients compared to the control group, (P<0.001) whereas, TIBC was significantly higher in the control group in comparison to thalassemic patients (P<0.001). (Table 2)

Haptoglobin &Malondialdehyde (MDA):

The study also revealed significant decrease in mean serum haptoglobin level in βTM patients than in controls (Mean ± SD 21.74±23.53 and 96.84±30.19 mg/dl respectively), (P <0.001), while the mean serum MDA level was significantly higher in thalassemia patients than control group (Mean ± SD 3.47±1.90 and 0.90±0.28 nmol/l respectively), (P<0.001). (Table 3).

Haptoglobin polymorphism

There was higher incidence of Hp 2-2 (56%) than other types Hp2-1 (30%) and Hp1-1 (14%) in thalassemic group with allele frequency of 71% for Hp-2 and 29% for Hp-1, while the control group showed more prevalence of Hp2-1(44%) followed by Hp2-2 (36%) then Hp1-1(20%) with allele frequency of 58% for Hp2 and 42% for Hp1. (Table 4)

Relation between haptoglobin, MDA, and ferritin with haptoglobin genotype in the thalassemic group.

The mean serum level of MDA was significantly higher in Hp2-2 genotype patients (Mean ± SD 3.92 ± 1.97) in comparison with the Hp1-1 genotype (Mean ± SD 2.31 ± 1.27). (p≤ 0.05), while mean serum haptoglobin levels were significantly lower in Hp2-2 genotype (Mean ± SD 18.65 ± 23.59) compared with that of Hp1-1 genotype (Mean ±SD 41.76 ± 30.17) (P ≤ 0.05). However mean serum ferritin was higher in thalassemic patients with the Hp2-2 genotype than in those with Hp1-1 and Hp2-1, but did not reach the significant value, (Mean ± SD 3502.8±2314.6, 3028.7±2093.5 and 3005.4±2055.7 respectively). (Table 5)

Table 1: Hematological data of βTM patients and the control group

Item	Cases (n=50)	Control (n=25)	P-value
Hb (gm/dl)			
Min. – Max	3.50-10.0	11.70-14.70	
Mean ± SD.	6.69±1.22	13.18±0.89	<0.001*
Median	6.65	13.0	
PCV (%)			
Min. – Max	9.90-30.50	35.0-45.60	
Mean ± SD.	21.19±3.94	40.97±3.02	<0.001*
Median	21.15	41.50	
MCV (fl)			
Min. – Max	53.40-88.20	75.0-100.0	
Mean ± SD.	72.73±8.67	82.28±4.98	<0.001*
Median	74.25	81.0	
MCH (pg)			
Min. – Max	16.30-27.70	20.40-32.30	
Mean ± SD.	23.12±3.18	28.65±2.63	<0.001*
Median	23.95	28.60	
MCHC (gm/dl)			
Min. – Max	23.70-38.10	30.10 – 36.0	
Mean ± SD.	31.93±2.75	32.34 ± 1.57	0.406
Median	32.70	32.0	
RBCs ($x10^{12}$/l)			
Min. – Max	1.35-4.50	4.28-5.70	
Mean ± SD.	2.94±0.71	5.03±0.38	<0.001*
Median	2.86	5.10	
Retic count($x10^9$/l)			
Min. – Max.	54.0 – 364.0	22.0 – 77.0	
Mean ± SD.	124.66 ± 69.43	39.44 ± 15.15	<0.001*
Median	98.0	43.0	
WBCs ($x10^9$/l)			
Min. – Max	3.84 – 9.73	4.0-9.80	
Mean ± SD.	6.64 ± 1.65	6.83±1.64	0.744
Median	6.55	7.10	
Plts ($x10^9$/l)			
Min. – Max	133.0-1056.0	131.0-455.0	
Mean ± SD.	457.28±263.80	243.80±89.33	<0.001*
Median	354.50	225.0	

Hb: hemoglobin, PCV: packed cell volume, MCV: mean corpuscular volume, MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; RBCs: red blood cells count; Retic count: reticulocyte count; WBCs: white blood cells count; Plts: Platelets count

*: Statistically significant at $p \leq 0.05$

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Table 2: Iron profile in thalassemic &control groups

Item	Cases (n = 50)	Control (n = 25)	P value
Serum iron (µmol/l)			
Min. – Max	22.40-64.40	10.70-23.30	
Mean ± SD.	42.30±9.81	15.53±2.83	<0.001*
Median	41.20	15.0	
TIBC (µmol/l)			
Min. – Max	33.50-64.40	42.90-62.80	
Mean ± SD.	41.72±7.87	51.34±5.11	<0.001*
Median	38.75	50.50	
TS (%)			
Min. – Max	38.0-160.0	17.0-47.0	
Mean ± SD.	106.66±33.28	30.88±7.42	<0.001*
Median	112.0	30.0	
Serum ferritin (µg/l)			
Min. – Max	890.0-9830.0	30.0-210.0	
Mean ± SD.	3287.22±2180.96	95.04±46.40	<0.001*
Median	2705.0	82.0	

TIBC: total iron binding capacity; TS: transferrin saturation
*: Statistically significant at $p \leq 0.05$

Table 3: Comparison between the two studied groups according to serum haptoglobin& MDA

Item	Cases (n = 50)	Control (n = 25)	P value
Serum haptoglobin (mg/dl)			
Min. – Max	1.90-99.0	48.50-158.0	
Mean ± SD.	21.74±23.53	96.84±30.19	<0.001*
Median	10.20	90.0	
Serum MDA (nmol/l)			
Min. – Max	1.10-9.10	0.20-1.30	
Mean ± SD.	3.47±1.90	0.90±0.28	<0.001*
Median	3.25	0.80	

MDA: malondialdehyde
*: Statistically significant at $p \leq 0.05$

Table 4: Haptoglobin Polymorphism in the studied groups

Item	Cases (n = 50)		Control (n = 25)		χ^2	P value	OR	95%CI	
	No.	%	No.	%				LL	UL
Haptoglobin									
Hp1-1	7	14.0	5	20.0	0.446	0.519	1.000	-	-
Hp2-1	15	30.0	11	44.0	1.442	0.230	0.974	0.243	3.897
Hp2-2	28	56.0	9	36.0	2.667	0.102	2.222	0.564	8.759
Hp 0-0	0	0.0	0	0.0	-	-	-	-	
Allele frequency									
Hp1	29	29.0	21	42.0	2.535	0.111	1.000	-	-
Hp2	71	71.0	29	58.0			1.773	0.873	3.601

χ^2: Chi-square test

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Table 5: Relation between MDA, haptoglobin, and ferritin with haptoglobin genotype in the thalassemic group.

Item	Haptoglobin genotype			P value
	Hp1-1 (n=7)	Hp2-1 (n=15)	Hp2-2 (n=28)	
Serum MDA (nmol/l)				
Min. – Max Mean ± SD. Median	1.10 – 4.60 2.31 ± 1.27 1.90	1.10 -7.60 3.17 ± 1.80 2.70	1.10 – 9.10 3.92 ± 1.97 3.45	≤ 0.05
Sig. bet. Grps. #	1-1 with 2-2*			
Serum haptoglobin (mg/dl)				
Min. – Max Mean ± SD. Median	5.0 – 84.0 41.76 ± 30.17 29.60	2.80 – 49.50 18.17 ± 15.51 12.0	1.90 – 99.0 18.65 ± 23.59 7.0	≤ 0.05
Sig. bet. Grps. #	1-1 with 2-2*			
Serum ferritin (µg/l)				
Min. – Max Mean ± SD. Median	1200 – 7000 3028.7±2093.5 2400.0	900 – 8400 3005.4±2055.7 2600.0	890 – 9830 3502.8±2314.6 3091.5	0.608

MDA: malondialdehyde
#: Pair-wise comparison was done using the Whitney test
*: Statistically significant at $p \leq 0.05$.

DISCUSSION

Thalassemia has emerged as a significant problem in clinical practice. Analysis of the molecular principles of the different types of thalassemia and the phenotype/genotype relationships have made great advances in understanding the processes behind the phenotypic variability in β-thalassemia **[16]**. This phenotypic variability is mainly due to genetic factors which still have been incompletely defined. In addition, little is known about the phenotypic heterogeneity complications of thalassemia and its pathophysiology. Repeated blood transfusions are necessary for the survival of thalassemia patients, but lead to excessive iron overload, altered trace element levels, and antioxidant enzymes, all of which may cause oxidative stress **[17]**.

Nitric oxide (NO) can oxidize heme, the free hemoglobin molecule carrying the iron moiety, causing oxidative stress-mediated damage via hydrogen peroxides and lipids. Plasma Haptoglobin (HP) binds to free hemoglobin to scavenge it from oxidation, possibly halting additional heme-mediated nitric oxide damage, iron renal excretion, and additional oxidative damage **[18]**.

In the current study, a significant decline in mean serum haptoglobin level in βTM patients compared to the control group was observed. Ragab et al **[19]** confirmed our results. They found that thalassemic children had intense Hp depletion when compared to the control group. They concluded that the presence of ineffective erythropoiesis as well as hemolysis results in a decrease in the level of plasma haptoglobin. Regardless of the hemolysis site (extravascular or intravascular) or the existence of concordant inflammation, its depletion is an indication of the rapid diagnosis of enhanced destruction of red cells. Thus, Hp depletion in patients with thalassemia is assigned to hemolysis and ineffective erythropoiesis.

MDA is a lipid peroxidation and oxidative stress marker that is produced as the final product of the oxygenation of polyunsaturated fatty acid **[5]**. Our results revealed a significant increase in MDA levels in thalassemia patients compared to controls which reflects the patient's state of oxidative stress. This agrees with Atmakusuma et al **[20]** who demonstrated that thalassemia patients show increased MDA levels compared to healthy controls reflecting the state of oxidative stress of these patients. In addition, Basu et al **[21]** reported that Malonaldehyde (MDA) is increased significantly in both transfusion and non-transfusion-dependent β-thalassemia. Regardless of severity and blood transfusion dependency, they signified MDA as a reliable surrogate indication of iron overload in β-thalassemia. As a result, it can be utilized

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instead of ferritin as a predictive biomarker of management, particularly in the consequence of chelation therapy.

In the present study, 56% of thalassemic patients had the Hp2-2 genotype, while in the control group Hp2-1 genotype predominated. Our results agree with Melamed-Frank et al, **[22]** who reported that Hp2-2 persons are more susceptible to oxidative stress than Hp1-1 and Hp2-1 persons. Moreover, our results showed that Hp2-1 and Hp2-2 genotypes are associated with lower serum haptoglobin levels while the genotype Hp1-1 is linked with the highest serum haptoglobin level. This result was confirmed by Van et al **[23]** who stated that persons with the Hp 2-2 phenotype have a lower reference range for haptoglobin concentration than those with the Hp 1-1 and Hp 2-1 phenotypes.

Additionally, Kasvosve et al **[24]** described that the reference values of haptoglobin depend on the Hp phenotype. In the same context, Imrie et al [25] reported that Hp levels were related to Hb levels, Hp genotype, splenomegaly, and age. Additionally, it appears that the rate of free hemoglobin clearance from circulation is phenotype-dependent as shown by Van et al **[23]** hence the clearance of the Hp1-1-Hb complex is faster than that of the Hp2-2Hb complex. Thus, the lower levels of serum haptoglobin in thalassemic patients could be attributed to the presence of different polymorphisms of the haptoglobin gene. Kaiser et al **[7]** found that people with Hp2-2 have the lowest concentrations of Hp, while those with Hp1-1 have the highest, and the ones with Hp2-1 have concentrations that fall somewhere in between.

Our study also revealed that thalassemic patients with Hp2-2 have the highest serum ferritin than other phenotypes which agrees with Ragab et al **[19]** who demonstrated that Hp2-2 genotype patients had higher serum ferritin compared to their analogs. When coupled to Hp 2-2 rather than Hp 1-1 or Hp 2-1, the Hb-Hp complex is more effectively internalized by CD163 receptors. Therefore, it was hypothesized that people with the Hp 2-2 phenotype experience more oxidative stress as a result of higher iron buildup in macrophages **[26]**.

On the other hand, we demonstrated that patients with the Hp2-2 phenotype had the highest level of MDA when compared to the other phenotypes. Our results corroborate the findings of Blum et al **[27]** who stated that the Hp-1 protein is allied with enhanced production of the antioxidant cytokine IL 10, whereas the Hp-2 protein is accompanied by an excess of oxidative active iron production. Subsequently, the Hp1 phenotype has anti-inflammatory and antioxidant properties. Moreover, Awadallaha et al **[28]** reported that upon formation of the Hp-Hb complex, it is rapidly quenched from the circulation and hunted by the macrophages and hepatocytes CD163 receptors.

In the current study, lower levels of serum haptoglobin could be interpreted by the presence of anti-Hp antibody which needs to be further studied. The risk of producing anti-Hp antibodies by blood transfusion has been investigated by Shimada et al **[29]** who reported that haptoglobin deficiency was associated with Hp IgG antibodies and those subjects could experience fatal anaphylactic non-hemolytic transfusion reactions. They also reported that the Hp-2 allele has a superior ability to form antibodies than the Hp-1 allele. It is worth mentioning that our results revealed higher Hp-2 allele frequency than Hp-1. Previous studies suggest that Hp- 1 allele may be beneficial from a biological standpoint due to its smaller size, faster Hb exportation, and increased antioxidative capabilities, so Hp 1-1 can be taken up by cells faster and pass the blood-brain barrier. On the other hand, the Hp-2 genotype is associated with a lower Hp concentration and a diminished competency to bind to Hb **[30]**.

CONCLUSION

We found functional variations in the antioxidant ability of different haptoglobin proteins towards hemoglobin, proposing that those with haptoglobin 1-1 protein may have better antioxidant protection than those with Hp 2-2 protein. Haptoglobin polymorphism and phenotypic variability have a major influence on oxidative stress in patients with thalassemia and those with the Hp 2-2 phenotype are more vulnerable to iron-steered oxidative stress than those with different phenotypes.

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Conflict of interest: None.

Ethical consideration

This study was approved by the Research Ethics Committee of Medical Research Institute, Alexandria University following principles of the

Declaration of Helsinki (IORG0008812). All studied patients signed for informed and written consent.

HIGHLIGHTS

- This study assessed the effect of haptoglobin gene polymorphism on phenotypic variability in thalassemia patients and its relation to iron overload and oxidative stress.
- The study revealed a higher incidence of haptoglobin 2-2 genotype in thalassemic patients. Serum levels of MDA & ferritin were higher and haptoglobin levels were lower in patients with Hp2-2 genotype.
- Haptoglobin polymorphism and phenotypic variability have a major influence on oxidative stress in patients with thalassemia and those with the Hp 2-2 phenotype are more vulnerable to iron-steered oxidative stress than those with different phenotypes.

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